This application claims the benefit of provisional application U.S. Serial No. 60/260,210 filed January 9, 2001.

Technical Field of the Invention

The invention relates to the field of clinical and analytical assays of biological products. In particular it relates to the field of immunoassays.

Background of the Invention

Islet neogenesis associated protein (INGAP) is a product of a gene expressed in regenerating hamster pancreas. INGAP gene is expressed in acinar cells, but not in islets. Western blot analysis demonstrates the presence of INGAP in Ilotropin, a protein extract isolated from cellophane wrapped pancreata, but not in extracts from control pancreata. A synthetic pentadecapeptide, corresponding to a region unique to INGAP, stimulates a 2.4-fold increase in [3H]thymidine incorporation into hamster duct epithelium in primary culture and a rat pancreatic duct cell line but has no effect on a hamster insulinoma tumor cell line. A portion of human INGAP gene has been cloned and appears to be highly homologous to the hamster gene. INGAP gene is a pancreatic gene expressed during islet neogenesis whose protein product is a constituent of Ilotropin and is capable of initiating duct cell proliferation, a prerequisite for islet neogenesis. J Clin Invest 1997 May 1;99(9):2100-9. There is a need in the art for tools for measuring INGAP to enable and facilitate the full exploitation of this important protein for fighting human disease, such as diabetes types I and II.

Summary of the Invention

It is an object of the invention to provide methods for assaying INGAP protein in a test sample.

It is an object of the invention to provide antibodies for assaying INGAP protein in a test sample.

One or more of the following embodiments provides these and other objects of the invention. In one embodiment of the invention a method is provided for assaying INGAP in a test sample. A solid support comprising bound antibodies is contacted with a test sample. The antibodies specifically bind to an amino acid sequence FLSWVEGEESQKKLPSSRITC (SEQ ID NO: 1) of INGAP protein. INGAP protein in the test sample binds to the solid support. Test sample which has not bound to the solid support is removed. The solid support is contacted with a quantity of a labeled INGAP molecule such that all or a portion of the quantity of labeled INGAP molecule binds to the solid support. Labeled INGAP molecule which has not bound to the solid support is removed. The amount of labeled INGAP molecule bound to the solid support is determined. Labeled INGAP molecule bound to the solid support is inversely related to INGAP protein in the test sample.

According to another embodiment of the invention a method is provided for assaying INGAP in a test sample. Antibodies which specifically bind to an amino acid sequence FLSWVEGEESQKKLPSSRITC (SEQ ID NO: 1) of INGAP protein are contacted with a test sample which may contain INGAP protein, and a labeled INGAP molecule. The amount of labeled INGAP molecule bound to the antibodies is determined. The amount of labeled INGAP molecule bound to the antibodies is inversely related to INGAP protein in the test sample.

According to another embodiment of the invention an isolated antibody is provided which specifically binds to an amino acid sequence FLSWVEGEESQKKLPSSRITC as shown in SEQ ID NO: 1.

These and other embodiments of the invention which are described in more detail

below, provide the art with a quantitative means of determining INGAP in culture media, biological tissues, and in biological fluids.

Brief Description of the Drawings

Fig. 1 is a schematic of an assay according to the present invention. INGAP detection antibody which is specifically immunoreactive with INGAP residues 20-40 can be attached to a solid support. A sample which contains INGAP is contacted with the solid support and the antibodies capture the INGAP. Unoccupied INGAP-binding sites on the solid support are then contacted with and occupied by a tagged INGAP molecule. The amount of tagged INGAP molecules captured on the solid support is determined, from which the amount of untagged INGAP in the test sample is calculated.

Fig. 2 shows a dose displacement curve using INGAP peptide (residues 20-40; "INGAP-LY") and recombinant INGAP.

Fig. 3 shows reproducible peptide displacement curves for six individual determinations of standards.

Fig. 4 shows reproducible peptide displacement curves for six individual determinations of standards at a later time point than in Fig. 3.

Fig. 5 shows the specificity of the assay. A peptide from a distinct region of INGAP (residues 104-118) was used as the tagged INGAP molecule.

Fig. 6 shows peptide regions of INGAP schematically.

Detailed Description of the Invention

It is a discovery of the present invention that antibodies directed to a particular portion of INGAP are able to bind to full-length INGAP and provide a reliable means of quantitation of INGAP in biological samples The antibodies are specific for the

particular portion of INGAP and other portions do not effectively compete for antibody binding.

The peptide immunogen FLSWVEGEESQKKLPSSRITC (SEQ ID NO: 1) consists of residue numbers 20-40 of full length INGAP protein. While applicants do not wish to be bound by any particular theory, it is believed that this portion is suitable because of its uniqueness to INGAP. Thus it is believed that other subfragments or overlapping fragments of at least 6, 8, 10, 12, or 14 contiguous amino acids up to about 30 amino acids would be useful. Applicants note that a peptide consisting of residues 104-118 did not successfully compete with full length INGAP for binding to the antibodies.

Assays of the invention can be performed in solution/suspension or on a solid support. Suitable solid supports include without limitation: microtiter dishes, filters, nylon, membranes, plastics, column packing materials, beads. Typically antibodies are applied to a surface in a liquid and the liquid is removed by drying. Alternatively, solid substrate or support can be derivatized to covalently bind to the antibodies. Such techniques are known in the art and can be selected as is convenient by one of ordinary skill in the art.

Test samples can be samples from cells, tissues, organs, or bodily fluids of a human or other test mammal. Samples can also be fermentation broths, or cell lysates from recombinant or natural cells which produce INGAP. Samples can be pretreated to remove fractions which do not contain INGAP. This can be done by specifically removing other components or by purifying and selecting for INGAP in samples.

Antibodies employed preferably have been produced by immunizing with a peptide according to SEQ ID NO: 1: FLSWVEGESQKKLPSSRITC. However, any peptide having at least six of such contiguous amino acids can be useful. Thus the

following peptides or polypeptides containing such peptides can be contemplated as immunogens.

FLSWVE (SEQ ID NO: 5)

LSWVEG (SEQ ID NO: 6)

SWVEGE (SEQ ID NO: 7)

WVEGES (SEQ ID NO: 8)

VEGESQ (SEQ ID NO: 9)

EGESQK (SEQ ID NO: 10)

GESQKK (SEQ ID NO: 11)

ESQKKL (SEQ ID NO: 12)

SQKKLP (SEQ ID NO: 13)

QKKLPS (SEQ ID NO: 14)

KKLPSS (SEQ ID NO: 15)

KLPSSR (SEQ ID NO: 16)

LPSSRI (SEQ ID NO: 17)

PSSRIT (SEQ ID NO: 18)

SSRITC (SEQ ID NO: 19)

Peptides can be parts of larger molecules. For example, protein adjuvants such as keyhole limpet hemocyanin can be conjugated to peptides to enhance immunogenicity. Antibodies can be polyclonal or monoclonal. They can be produced by any technology known in the art, including hybridomas, cloning, and phage display. Antibodies can be screened and selected and purified as desired using any technique known in the art. They can be produced in humans, goats, rabbits, mice, etc. They can be the product of an antibody encoded by any species, whether produced in that species or a heterologous species.

The assay of the present invention is a competitive assay in which unlabeled analyte (INGAP) in a sample competes with a labeled analyte for binding to the antibody. The labeled analyte can be full-length INGAP or an appropriate INGAP peptide which binds to the antibody. The labeled analyte can be, *e.g.* radiolabeled, fluorescently labeled, or enzymatically labeled. The label can be covalently attached to the analyte or can be attached via a strong binding interaction, *e.g.*, avidin/biotin.

Other peptide sequences can be used in place of residues 20-40 of INGAP. These include peptides comprising residues 104-118 (IGLHDPSHGTLPNGS; SEQ ID NO:2), 139-152 (IAADRGYCAVLSQK; SEQ ID NO:3), and 151-164 (QKSGFQKWRDFNCE; SEQ ID NO:4). As stated above, subfragments of as little as six residues can be used as immunogens, and can be fused or conjugated other proteins or adjuvant molecules. Use of such peptides requires use of the corresponding antibody in the assay.

Examples

I. General Methodology:

INGAP specific antibody is coated at the required concentration onto a solid matrix. Following coating, the matrix is washed twice and remaining non-specific protein binding sites blocked by incubation in an appropriate blocking buffer. The matrix is washed in a wash buffer and samples or standards to be assayed are incubated with the matrix. Washing of the matrix removes unbound INGAP and the remaining exposed antibody sites are probed by incubating the matrix with an INGAP reporter construct (details below). Quantitation of the INGAP reporter construct is achieved

with an appropriate detection technique. Displacement of signal provides a measure of INGAP in test material.

III. Details of the current assay:

50 :l of INGAP(residues 20-40) antibody in PBS is added to wells of a 96 well flat bottom MAXISORB™ plate (NUNC). To control wells is added 50 :l of PBS. The plate is incubated at 37°C in a dry incubator for 3 hrs. Each well of the plate is washed twice with 200 :l of PBS and tapped dry before being filled with 300 :l of 2% BSA-PBS and incubated overnight at 4°C to block free protein binding sites in each well. After the overnight incubation each well of the plate is washed twice with 200 :l of PBS before up to 200 :l of sample or known dilutions of rINGAP or INGAP-peptide is added to the appropriate wells. The highest dilution of the known protein is also added to the wells that were not coated with anti-INGAP(residues 20-40). The samples are left to bind for 2 hours at 37°C in a dry incubator. The contents of each well is removed and the wells washed thrice in HBHA buffer (Hank's balance salt solution, 0.5mg/ml BSA, 0.1% NaN3, 20mM HEPES, pH7.0) before the addition of 100:l of culture supernatant from INGAP-fusion protein-expressing cells. Following a further incubation at 37°C for 90 min. the wells were washed thrice with 200 :l of HBHA and tapped dry. The amount of INGAP fusion protein bound in each well was determined by the addition to each well of 100:l of chromogenic substrate for the fusion protein. The development of color is determined by reading the absorbance of each well. The displacement of signal obtained using the known concentrations of INGAP (or peptide) is used to generate a standard curve in an excel spreadsheet from which INGAP concentrations in the samples are calculated.

IV. Generation of INGAP fusion proteins:

Cloned INGAP cDNA was subjected to PCR using 3' and 5' primers which introduced desired restriction sites 3' and 5' respectively to allow the insertion of INGAP into cloning vectors in the correct reading frame to form fusion proteins. Restriction digestion of the vector and PCR adjusted INGAP genes followed by ligation of the purified products, resulted in INGAP fusion constructs.

IV.1 INGAP-fusion protein with enzyme

Cloned INGAP gene was subject to PCR using the 3' primer (5'GCGAAGATCTGACCTTGAATTTGCAGAT3'; SEQ ID NO: 20) and 5' primer (5'CGCCCAAGCTTACCATGATGCTTCCCATGACCCTC3'; SEQ ID NO: 21) which introduced the restriction sites BglII and HindIII 3' and 5' respectively onto the INGAP gene. This allowed the insertion of INGAP into a vector in the correct reading frame. Restriction digestion of the vector and PCR adjusted INGAP with BglII and HindIII was followed by ligation of the purified products. The resulting vector expresses the fusion protein.

IV.2 INGAPMAT fusion protein with enzyme

Cloned INGAP gene without the signal sequence (INAGPMAT) was subjected to PCR using the 3' primer (5'GCCGCTCGAGCTAGACCTTGAATTTGCAGAT3'; SEQ ID NO: 22) and 5' primer (5' GCCGAAGATCTGAAGAATCTCAAAAGAAACTG3'; SEQ ID NO: 23) which

introduced the restriction sites XhoI and BglII 3' and 5' respectively onto the INGAPMAT gene. This allowed the insertion of INGAPMAT into a vector in the correct reading frame. Restriction digestion of the vector and PCR adjusted INGAPMAT with XhoI and BglII was followed by ligation of the purified products. The resulting vector expresses the fusion construct.

IV.3 INGAP peptide fusion with enzyme

Cloned INGAP gene was subjected to PCR using the 3' primer (5' GCCGCTCGAGCTAACTTCCGTTGGGTAGTGTACC3'; SEQ ID NO: 24) and 5' primer (5'GCCGAAGATCTATTGGACTCCATGATCCCTCA3'; SEQ ID NO: 25)) which introduced the restriction sites XhoI and BglII 3' and 5' respectively onto a partial sequence the INGAP gene. This allowed the insertion of INGAP peptide 104-118 into the vector in the correct reading frame. Restriction digestion of the vector and PCR adjusted INGAP with XhoI and BglII was followed by ligation of the purified products. The resulting vector expresses the fusion construct comprising marker protein and INGAP peptide 104-118.

IV.4 INGAP fusion

Cloned INGAP gene was subjected to PCR using the 3' primer (5'CGCCCAAGCTTGACCTTGAATTTGCAGAT3'; SEQ ID NO: 26) and 5' primer (5'GCCGAAGATCTATGATGCTTCCCATGACCCTC3'; SEQ ID NO: 27) which introduced the restriction sites XhoI and BglII 3' and 5' respectively onto the INGAP gene. This allowed the insertion of INGAP into the vector in the correct

reading frame. Restriction digestion of the vector and PCR adjusted INGAP with XhoI and BglII was followed by ligation of the purified products. The resulting vector expresses the fusion construct of INGAP and a fluorescent marker protein.

V. Production of INGAP fusion proteins

Using lipofectamine (Gibco BRL), 10 :g of the INGAP-fusion protein expression vector was transiently transfected into 293T cells. At time intervals following transfection the culture supernatant was harvested from the cells and assayed for INGAP fusion protein expression. Either purified concentrates, or dilutions, of the expressed fusion protein are used in INGAP detection assays.

Example 1

By using known concentrations of the INGAP peptide (residues 20-40) a dose displacement curve was generated and the data generated subsequently transformed to display percentage inhibition. Figure 2. The curve clearly displays dose-dependant saturable-binding. Test dilutions of a recombinant INGAP were assayed to determine the actual concentration of recombinant INGAP in the preparation. Using the INGAP 20-40 peptide curve the equivalent molar concentration of recombinant INGAP and INGAP 20-40 peptide was determined. The curves shown are superimposed at their calculated molar concentrations. These data provide an accurate determination of recombinant INGAP concentration.

Example 2

INGAP 20-40 and INGAP 114-118 were compared in their ability to compete for binding to the antibodies of the invention. Figure 5 shows that only

INGAP 20-40 functions in this assay. The assay was performed at two antibody coating concentrations, 7 and 10 ug/ml, to explore further the potential for non-specific interactions.